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The influence of macrophage growth factors on Theiler's Murine Encephalomyelitis Virus (TMEV) infection and activation of macrophages

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ABSTRACT

Macrophages are common targets for infection and innate immune activation by many pathogenic viruses including the neurotropic Theiler's Murine Encephalomyelitis Virus (TMEV). As both infection and innate activation of macrophages are key determinants of viral pathogenesis especially in the central nervous system (CNS), an analysis of macrophage growth factors on these events was performed. C3H mouse bone-marrow cells were differentiated in culture using either recombinant macrophage colony stimulating factor (M-CSF) or granulocyte–macrophage colony-stimulating factor (GM-CSF), inoculated with TMEV (BeAn) and analyzed at various times thereafter. Cytokine RNA and protein analysis, virus titers, and flow cytometry were performed to characterize virological parameters under these culture conditions. GM-CSF-differentiated macrophages showed higher levels of TMEV viral RNA and proinflammatory molecules compared to infected M-CSF-differentiated cells. Thus, GM-CSF increases both TMEV infection and TMEV-induced activation of macrophages compared to that seen with M-CSF. Moreover, while infectious viral particles decreased from a peak at 12 h to undetectable levels at 48 h post infection, TMEV viral RNA remained higher in GM-CSF- compared to M-CSF-differentiated macrophages in concert with increased proinflammatory gene expression. Analysis of a possible basis for these differences determined that glycolytic rates contributed to heightened virus replication and proinflammatory cytokine secretion in GM-CSF compared to M-CSF-differentiated macrophages. In conclusion, we provide evidence implicating a role for GM-CSF in promoting virus replication and proinflammatory cytokine expression in macrophages, indicating that GM-CSF may be a key factor for TMEV infection and the induction of chronic TMEV-induced immunopathogenesis in the CNS.

1. Introduction

Bone-marrow derived monocytes/macrophages are targets for infection and replication by viruses such as Human Immunodeficiency virus (HIV) [9,12,35], Dengue [50,58], Ebola [16], RSV, Influenza A and Theiler's Murine Encephalomyelitis (TMEV) [42,54]. Macrophages are composed of very heterogeneous and plastic populations, whose differentiation from monocytes is driven primarily by two macrophage growth factors: macrophage-colony stimulating factor (M-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) [51,55,61]. The state of macrophage differentiation at the time of virus infection is important, as virus interactions with different macrophage subpopulations can result in alternative disease outcomes [68].

M-CSF and GM-CSF exert different influences on phenotype and function of macrophages [55]. Macrophages differentiated with these factors are relatively quiescent until triggered by appropriate microbe-derived ligands (pathogen associated molecular patterns, PAMPs) that stimulate various pathogen recognition receptors (PRRs) expressed on macrophages [4]. For instance, M-CSF has been shown to prime macrophages to respond to PAMPs with an anti-inflammatory profile including IL-10 secretion [23]. In contrast, GM-CSF primes monocytes to respond with an inflammatory macrophage phenotype [23,32]. Inflammatory macrophages express proinflammatory cytokines such as IL-12, IL-23, TNF-α, IL-1α, IL-1β, and CCL2 [13,46,47,69] when exposed to various PAMPs [60]. In addition to proinflammatory cytokine production in macrophages, GM-CSF increases rates of nitric oxide production, phagocytosis, antigen presentation, cell survival, and proliferation in response to PAMPs [6]. Based on these differences, studies have shown that GM-CSF and M-CSF differentiated macrophages can shape the outcome of inflammation-driven diseases by affecting the

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polarization states of macrophages [8,32,34,52].

Macrophages have been shown to play an important role in virus-induced CNS disease [1,19,73]. Monocytes infected in the periphery are able to cross the blood brain barrier by the "Trojan horse" pathway, and differentiate into macrophages once in the tissue under the influence of local factors including macrophage growth factors, cytokines and microbial materials. Of particular relevance to the present studies, both M-CSF and GM-CSF are produced within the CNS parenchyma during CNS virus infection by microglia and astrocytes respectively [27,56]. Moreover, GM-CSF produced by infiltrating T cells provides an additional stimulus to monocyte differentiation in the CNS [20,63,66]. Under these influences, monocyte-derived macrophages produce a distinct array of proinflammatory cytokines, chemokines and toxic molecules that either promote anti-microbial immunity or, if dysregulated, damage CNS tissue [38].

The neurotropic virus, TMEV, is a murine single-stranded RNA picornavirus. Attenuated forms of the virus are used to study biphasic demyelinating disease as a model for the human demyelinating disease, multiple sclerosis [17,70]. In the TMEV model, viral RNA copies have been detected in high numbers in the spinal cord up to six months after infection despite the lack of infectious virions, indicating a propensity for the persistence of TMEV genomes in the CNS [41,53,73]. An explanation for the disparity between viral RNA and infectious virus has not yet been elucidated during TMEV persistence [25], but chronically elevated levels of viral RNA represent a source of persistent innate immune activation and potential reactivation of viral production. Previous studies have shown that TMEV infects and replicates in monocytes/macrophages [11,41,42,54]. More specifically, evidence suggests that TMEV replicates in terminally differentiated pro-inflammatory macrophages, but not undifferentiated macrophages [39,40,42]. In Src homology region 2 domain-containing phosphatase 1 (SHP-1)-deficient mice, our lab established that intracranial or peritoneal infection of suckling mice with the attenuated BeAn strain of TMEV resulted in macrophage-mediated CNS demyelinating disease that was not seen in wild type (WT) mice [10,11,54], demonstrating a key role of innate immune activation of macrophages in the demyelinating process. Our previous *in vivo* studies found that monocyte-derived macrophages ($CD45^{hi}CD11b + F4/80^{lo}$) infiltrated the CNS of SHP-1-deficient mice at significantly higher levels than wild type mice during TMEV infection, and that the infiltrating SHP-1-deficient macrophages had a 5-fold increase of TMEV RNA compared to wild type mice, indicating TMEV replicated at a higher rate in SHP-1-deficient macrophages [10]. Our studies also found that the TMEV-infected SHP-1-deficent macrophages are more M1-like, with significantly higher levels of IL-6 and IL-1β compared to wild type TMEV-infected macrophages [10,71].

Since both GM-CSF and M-CSF have been shown to play different roles in disease and macrophage differentiation and function including in the CNS [7,44,55,68], it was important to probe the influence these growth factors on TMEV infection, RNA persistence, and innate activation of macrophages *in vitro*. The latter was particularly relevant to our previous *in vivo* studies suggesting that the pathogenic infiltrating macrophages were M1-like, and therefore might be variably influenced by these factors. Our analysis shows that GM-CSF- and M-CSF-differentiated macrophages develop unique phenotypes that respond to TMEV infection in distinct ways. In particular, GM-CSF differentiated macrophages infected with TMEV had higher levels of viral RNA, infectious virus, and proinflammatory molecules compared to infected M-CSF-differentiated cells. Importantly, while both M-CSF and GM-CSF differentiated macrophages ceased to produce infectious TMEV viral particles over time, TMEV genomes uniquely persisted in GM-CSF-differentiated macrophages suggesting a pathogenic role for GM-CSF in TMEV persistence and inflammation seen in CNS TMEV infections. Of particular interest, we found that the glycolytic rate of GM-CSF macrophages was significantly higher than in M-CSF macrophages, suggesting that TMEV required glycolysis in inflammatory macrophages for efficient replication. This observation may provide a potential mechanism for increased TMEV replication and proinflammatory activity in GM-CSF-derived macrophages in TMEV-induced macrophage-mediated disease.

2. Materials and methods

2.1. Animals

Homozygous wild type mice were produced from congenic C3FeLe.B6 *a/a-Ptpn6/J* wild type mice (Jackson Laboratories, Bar Harbor, ME) All animal experiments were performed under approval from the Institutional Animal Care and Use Committee (IACUC) at SUNY Upstate Medical University.

2.2. Bone marrow-derived macrophages

Bone marrow-derived macrophages were prepared by cell harvest from femurs and tibias of two-to-three week old wild type mice. Cells were cultured in complete medium containing 10% fetal bovine serum (FBS; Tissue Culture Biologicals, Long Beach, CA; No. 101), 1% Penicillin Streptomycin (Corning Cellgro, Manassas, VA; No. 30-02 CI) in DMEM with 4.5 g/L glucose, L-glutamine and sodium pyruvate (Corning Cellgro, Manassas, VA; No. 10-013-CV). Medium was supplemented with 20 ng/ml recombinant mouse M-CSF or GM-CSF (R & D Systems, Minneapolis, MN; No. 416-ML-050 or 415-ML-050, respectively), and cells were differentiated into macrophages for 6 days at 37 °C.

2.3. Macrophage infections

BeAn TMEV was obtained from ATCC (Manassas, VA; No. VR-995) and propagated in BHK-21 cells (ATCC; No. CCL-10). Plaque assays were performed to determine viral titer as plaque-forming units per milliliter (PFU/ml). Bone marrow-derived macrophages were washed with serum-free DPBS and infected with BeAn TMEV at an MOI of 10 or mock infected with serum-free DMEM. Cells were gently agitated every 15 min, and after adsorption of TMEV for one hour at 37 °C virus was removed and cells were cultured in complete medium containing 20 ng/ml recombinant mouse M-CSF or GM-CSF. At 1, 12, 24 and 48 h time points supernatant and cells were spun down to collect adherent and nonadherent cells. Supernatants were removed and stored at −80 °C and cell pellets were suspended in RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) and stored at −80 °C until further processing for RNA extraction/analysis. For the kinetics study, bone-marrow-derived macrophages were grown, infected and collected as described at 1, 12, and 24 h post-infection. For the 2-DG assay, bone marrow derived macrophages were isolated, grown and inoculated with TMEV as previously described. After inoculation, 50 mM of 2-DG was added to the complete medium, and supernatants were removed 12 h.p.i. and stored at −80 °C.

2.4. RNA analysis

RNAs were purified from cell pellets by extraction using the RNeasy Mini Kit (Qiagen, Germantown, MD; No. 74104), and were analyzed by a custom designed Quantigene 2.0 Multiplex Assay (Affymetrix, Inc., Santa Clara, CA) for the 1, 12, and 24 h time points. The Affymetrix QuantiGene Plex 2.0 Assay (a multiplex bead-based assay) was used to measure the expression of 41 genes of interest (including 38 target genes and 3 reference genes). The plate was read using the BioRad BioPlex 200 instrument using settings of 100 µl volume; 60 s timeout; and 100 Bead Events/Bead regions. Fluorescent readings from blank wells were subtracted from fluorescent values for each mRNA of interest. Values exceeding background were then normalized to the geometric mean signal derived from three reference genes in each sample: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH),